Clinical onset and course, response to treatment and outcome in 24 patients with the cblE or cblG remethylation defect complemented by genetic and in vitro enzyme study data

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Abstract: BACKGROUND: The cobalamin E (cblE) (MTRR, methionine synthase reductase) and cobalamin G (cblG) (MTR, methionine synthase) defects are rare inborn errors of cobalamin metabolism leading to impairment of the remethylation of homocysteine to methionine. METHODS: Information on clinical and laboratory data at initial full assessment and during the course of the disease, treatment, outcome and quality of life was obtained in a survey-based, retrospective study from physicians caring for patients with the CblE or CblG defect. In addition, data on enzyme studies in cultured skin fibroblasts and mutations in the MTRR and MTR gene were analysed. RESULTS: In 11 cblE and 13 cblG patients, failure to thrive, feeding problems, delayed milestones, muscular hypotonia, cognitive impairment and macrocytic anaemia were the most frequent symptoms. Delay in diagnosis depended on age at first symptom and clinical pattern at presentation and correlated significantly with impaired communication abilities at follow-up. Eighteen/22 patients presented with brain atrophy or white matter disease. Biochemical response to treatment with variable combinations of betaine, cobalamin, folate was significant. The overall course was considered improving (n = 8) or stable (n = 15) in 96 % of patients, however the average number of CNS symptoms per patient increased significantly over time and 16 of 23 patients were classified as developmentally delayed or severely handicapped. In vitro enzyme analysis data showed no correlation with outcome. Predominantly private mutations were detected and no genotype- phenotype correlations evident. CONCLUSIONS: The majority of patients with the cblE and cblG defect show limited clinical response to treatment and have neurocognitive impairment.

DOI: https://doi.org/10.1007/s10545-014-9803-7

to treatment and outcome in 24 patients with the cblE or cblG remethylation defect complemented by genetic and in vitro enzyme study data. Journal of Inherited Metabolic Disease, 38(5):957-967.
DOI: https://doi.org/10.1007/s10545-014-9803-7

J Inherit Metab Dis (2015) 38:957–967
DOI 10.1007/s10545-014-9803-7

ORIGINAL ARTICLE

Clinical onset and course, response to treatment and outcome in 24 patients with the cblE or cblG remethylation defect complemented by genetic and in vitro enzyme study data

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Received: 6 September 2014 / Revised: 1 December 2014 / Accepted: 2 December 2014 / Published online: 20 December 2014

Abstract
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Methods Information on clinical and laboratory data at initial full assessment and during the course of the disease, treatment, outcome and quality of life was obtained in a survey-based, retrospective study from physicians caring for patients with the CblE or CblG defect. In addition, data on enzyme studies in cultured skin fibroblasts and mutations in the MTRR and MTR gene were analysed.
Results In 11 cblE and 13 cblG patients, failure to thrive, feeding problems, delayed milestones, muscular hypotonia, cognitive impairment and macrocytic anaemia were the most

Communicated by: Carlo Dionisi-Vici

Electronic supplementary material The online version of this article (doi:10.1007/s10545-014-9803-7) contains supplementary material, which is available to authorized users.

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frequent symptoms. Delay in diagnosis depended on age at first symptom and clinical pattern at presentation and correlated significantly with impaired communication abilities at follow-up. Eighteen/22 patients presented with brain atrophy or white matter disease. Biochemical response to treatment with variable combinations of betaine, cobalamin, folate was significant. The overall course was considered improving (n=8) or stable (n=15) in 96 % of patients, however the average number of CNS symptoms per patient increased significantly over time and 16 of 23 patients were classified as developmentally delayed or severely handicapped. In vitro enzyme analysis data showed no correlation with outcome. Predominantly private mutations were detected and no genotype– phenotype correlations evident.

Conclusions The majority of patients with the cblE and cblG defect show limited clinical response to treatment and have neurocognitive impairment.

Introduction

The cobalamin E (cblE, OMIM 236270) and cobalamin G (cblG, OMIM 250940) complementation groups are rare inborn errors of intracellular cobalamin (cbl) metabolism. The metabolic block results in impairment of remethylation of homocysteine (Hcy) to methionine (Met) which is catalysed by the cytosolic enzyme methionine synthase (*MTR*, gene locus 1q43, defective in the cblG defect) and its closely associated enzyme methionine synthase reductase (*MTRR*, gene locus 5p15.31, defective in the cblE defect) that maintains MTR in its functional reduced status. Both disorders follow an autosomal recessive inheritance pattern (Watkins et al 2002).

Until now, a number of case reports and small case series on a total of less than 50 patients with either the cblE or the cblG defect have been published. In the majority of published patients, initial symptoms developed in the first weeks of life or in early childhood (Zavadakova et al 2005).

The clinical patterns of the cblE and the cblG defect seem to be indistinguishable, except for a subgroup of patients homozygous for the c.1361C > T mutation in the *MTRR* gene without neurological involvement but exclusively macrocytic anaemia (Vilaseca et al 2003), but this question has not been formally addressed until now. In general, a pattern of haematological, neurological and ophthalmological symptoms is characteristic for the disorders. The most frequently reported symptoms are megaloblastic/macrocytic anemia or macrocytosis (Fowler et al 1997; Müller et al 2007; Richard et al 2013; Schuh et al 1984; Steen et al 1997; Vilaseca et al 2003; Zavadakova et al 2002, 2005; Carmel et al 1988; Harding et al 1997; Labrune et al 1999; Outteryck et al 2012); developmental delay, cognitive dysfunction or mental retardation (Fowler et al 1997; Müller et al 2007; Richard et al 2013;

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Rosenblatt et al 1985; Schuh et al 1984; Steen et al 1997; Zavadakova et al 2002, 2005; Carmel et al 1988; Kvittingen et al 1997; Outteryck et al 2012; Poloschek et al 2005); seizures (Müller et al 2007; Richard et al 2013; Steen et al 1997; Zavadakova et al 2002; Harding et al 1997; Kvittingen et al 1997); pathological eye movements including nystagmus and impaired visual acuity (Steen et al 1997; Zavadakova et al 2002; Kvittingen et al 1997; Outteryck et al 2012; Poloschek et al 2005) and failure to thrive and feeding difficulties (Müller et al 2007; Zavadakova et al 2002; Labrune et al 1999).

Microangiopathy and haemolytic uremic syndrome (HUS) which have often been described in other inborn errors of cobalamin metabolism such as the cblC defect have only very rarely been reported in the cblE or cblG defect (Labrune et al 1999; Palanca et al 2013). Other less frequently reported symptoms are pulmonary hypertension (Labrune et al 1999); leukopenia or pancytopenia (Palanca et al 2013; Schuh et al 1984); recurrent infections (Harding et al 1997); altered consciousness or microcephaly (Fowler et al 1997); hydrocephalus and/or white matter disease (Müller et al 2007; Palanca et al 2013; Zavadakova et al 2005; Outteryck et al 2012).

Treatment reportedly encompasses folic or folinic acid, betaine and a variety of cobalamin preparations (hydroxo-, cyano- or methylcobalamin). The biochemical parameters total Hcy (tHcy) and Met mostly respond well to treatment. However, evaluation of clinical treatment efficacy is complex due to the highly variable medication in the context of individual profiles of mutations, age at onset of symptoms, time to initiation of treatment and clinical presentation. Some authors found no response at all to treatment in their patients (Steen et al 1997; Palanca et al 2013). In a series of nine European cblE patients, the overall impact of treatment on neurodevelopmental disabilities is considered "at most moderate" (Zavadakova et al 2005). In other cases, treatment— especially when started early— was beneficial (Müller et al 2007).

The aims of the present study are

i) to compare clinical signs and symptoms between patients with the cblE or the cblG defect
ii) to investigate factors related to delay in diagnosis
iii) to describe the clinical presentation at onset and during the course of the disease in a larger series of patients with the cblE / G defect
iv) to identify parameters (e.g. age at initial symptoms, delay in diagnosis, treatment, biochemical/genetic parameters) correlating with outcome and quality of life as rated by the referring physician

Methods

This retrospective study was approved by the local ethics committee (Kantonale Ethikkommission Zürich; No. 2013-0012). Recruitment of patients was based on fibroblast cell lines which had been sent for diagnostic purposes to Basel/Zürich, Switzerland and in which the CblE or CblG defect had been proven by enzymatic studies including somatic and complementation analysis.

Survey

All physicians who had sent cell lines were asked to complete a survey on clinical symptoms at onset and during the course of the disease, outcome, burden of disease and treatment, biochemical data and mutations. Informed consent was obtained from patient(s) and/or the caregivers. The survey was developed on the basis of the literature on the cblE and cblG defect and expert discussions. Questions addressed clinical symptoms reported before as well as involvement of other organ systems, e.g. metabolic crisis, failure to thrive, feeding problems, vomiting, drowsiness or impaired consciousness, anaemia, seizures, developmental delay, eye disease and hearing loss, muscular hypotonia, movement disorder, impaired cognitive and speech development, psychiatric and neurological symptoms, thromboembolic events, affection of the liver, kidney or heart. Open questions asked for comments or invited to mention any other signs or symptoms observed in the patient and not addressed explicitly. In addition, physicians were asked to give an overall estimation of the degree of the clinical course, cognitive impairment, quality of life of the patient and his parents, and behavioural and social issues on 5-point Likert scales. (Survey provided as supplementary material.)

Cell cultures and in vitro enzyme studies

Data from diagnostic analyses, which had been performed earlier in cultured skin fibroblasts from the study patients, were included with informed consent from the patients/their parents and referring clinicians. At time of diagnosis, synthesis of methionine from [^{14}C]formate had been determined in intact fibroblasts as an indirect measure of methionine synthase activity as described earlier (Suormala et al 2004). For this assay cells were grown for 72 h in routine culture medium (basal medium) without and with added hydroxocobalamin (OH-Cbl, 1 mg/L medium) for evaluation of in vitro cobalamin responsiveness. Synthesis of the cobalamin coenzymes, methylcobalamin (MeCbl) the cofactor of methionine synthase and adenosylcobalamin (AdoCbl) the cofactor of methylmalonyl-CoA mutase, from [^{57}Co]cyanocobalamin in intact fibroblasts had been measured as described earlier (Suormala et al 2004).

Mutation identification

For patients 3, 4, 5, 8, 10, 15, 16, 17, 19, 21 and 23, results of externally performed molecular genetic studies were recorded.

For the remaining patients, molecular genetic analyses were performed in the Zürich laboratory as follows: Genomic DNA and total RNA were extracted from cultured patient fibroblasts samples using the QIAamp DNA Mini Kit and RNeasy Kit (Qiagen), respectively. To identify mutations, exons were amplified through PCR from genomic DNA using flanking intronic primers (primers available upon request) and subsequent sequencing by the ABI BigDye method (Life Technologies). In cases where no or only one mutation was found, or to confirm splicing defects, cDNA was analysed following synthesis from total RNA by RT-PCR using OneStep RT-PCR kit (Qiagen) with direct sequencing of RT-PCR products.

Statistical analysis

Data were analysed using SPSS statistical software for Macintosh, release 20 (SPSS Inc., Chicago, IL, USA). Analyses were performed with two-sided tests with a p value of<0.05 considered to be significant. Chi-squared analyses were used to compare nominal variables across diagnostic groups. Nonparametric Spearman–Brown rank correlations were calculated in order to examine associations between variables. McNemar tests were used to statistically compare signs and symptoms at onset and during the course.

Results

Patient characteristics

Physicians caring for 47 patients were contacted and invited to participate. In 21 cases, physicians did not respond or had no information on where patients were followed. In two cases, informed consent for participation was not available. Ultimately, data sets were included in this retrospective survey study from 11 patients (five males, six females) with the CblE defect and 13 patients (eight males, five females) with the CblG defect. Patients originated from Germany ($n=6$), Czech Republic ($n=5$), Austria, the UK and Spain (each $n=2$), Australia, Belgium, Greece, Italy, Slovakia, Slovenia, and Turkey (each $n=1$). Parental consanguinity (2nd degree cousins) was present in the Turkish patient only. Patient care was predominantly in the hands of metabolic specialists ($n=22$) and/or paediatric neurologists ($n=21$). The diagnosis of the cblE or cblG defect was established on the basis of symptom-oriented, selective metabolic screening in all patients and in no case by newborn- or family screening.

Comparison of clinical presentation between patients with the cblE or the cblG defect

Muscular hypotonia during the disease course was observed more frequently in patients with the cblG defect (84 to 40 %; $p=0.039$; Chi Square test). No other significant differences were observed between cblE and cblG patients concerning clinical presentation at onset or during the course of the disease (Chi Square test, data not shown). Due to this almost complete clinical overlap, all further analyses combined patients with both disorders.

Pregnancy and newborn period

Pregnancy complications were reported in two cases: Intrauterine growth retardation in one and reduced fetal movements in another patient. Mean gestational week at birth was 39.5 weeks; three children were born preterm at week 34, 36 and 37 respectively. Birth weight (mean 3234 g, range 1910–4100 g), length (mean 50.2 cm, range 45–55 cm) and head circumference (mean 34; range 31–37 cm) were within the normal, age adjusted percentile ranges. One patient presented with facial dysmorphic features, ureteral duplication and external hydrocephalus at birth.

Age at first clinical symptoms, time to treatment initiation and diagnosis

Median age at first symptoms was 3 months (mean 2 years; range 1 day to 20.7 years) (Fig. 1).

Median time from the first appearance of symptoms to proven diagnosis (by enzymatic/molecular genetic studies) was 1.4 years (range from 198 days to 11.7 years; mean 3.4 years). Since treatment was initiated as soon as the disease was suspected from metabolic workup, time between first symptoms and treatment initiation was shorter in all patients (median 3.3 months, mean 1.98 years, range 0 days to 10.8 years). Median age at treatment initiation was 9 months (mean 4 years, range 1.5 months to 21.3 years). Age at disease onset correlated significantly negative with feeding problems ($p<0.01$) and growth impairment ($p<0.05$) indicating that these symptoms were more frequent in early onset patients (Spearman rho, Supplementary Table 1).

Delay in diagnosis

Delay in diagnosis was shorter in patients who developed symptoms at an early age ($p<0.05$) and if feeding problems ($p<0.01$), drowsiness/impaired consciousness ($p<0.05$) and impaired growth ($p<0.05$) were present at disease onset (Spearman rho, Supplementary Table 1).

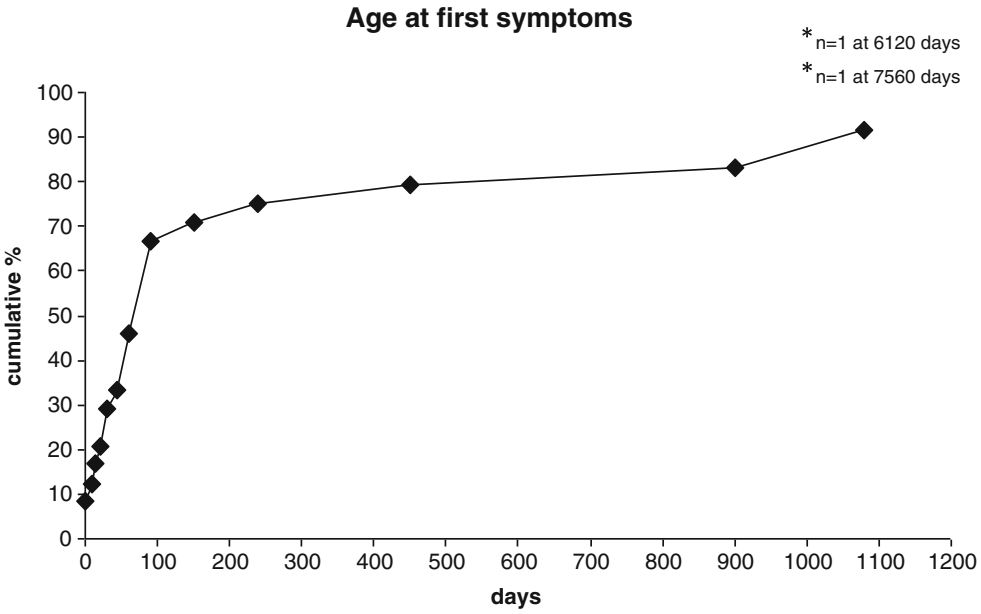

Fig. 1 Age at first symptoms (cumulative frequency) in 24 patients with the cblE and cblG defect

Clinical presentation at onset and during the course of the disease (Fig. 2)

One patient with severe neurological symptoms and hypertension had died at age 56 days. Mean age at follow-up was 11.9 years (range 12 months to 32 years, median 11.1 years).

Feeding problems, muscular hypotonia, cognitive impairment and delayed development as well as macrocytic anaemia were the most frequent symptoms. The comparison of the frequency of single clinical symptoms both at onset and during the course revealed no significant differences between both measurement points for any of the signs or symptoms (McNemar Test, data not shown). Clinical deterioration/crises following infections or similar triggers was not observed and the overall clinical course was considered improving in 33 %, stable in 63 % and deteriorating in 4 % of cases by the physicians. However, when combining parameters indicative for central nervous system (CNS) involvement and visual impairment (microcephaly, seizures, delayed achievement of developmental milestones, cognitive impairment, impaired speech, eye disease and psychiatric symptoms) to define a score for "CNS/visual involvement", the average number of symptoms per patient was significantly lower at onset with 2.41 compared to 3.87 during the course (paired *t*-test; t= −3.27, p=0.003) indicating a significant worsening over time. Eye problems and visual impairment were mainly due to

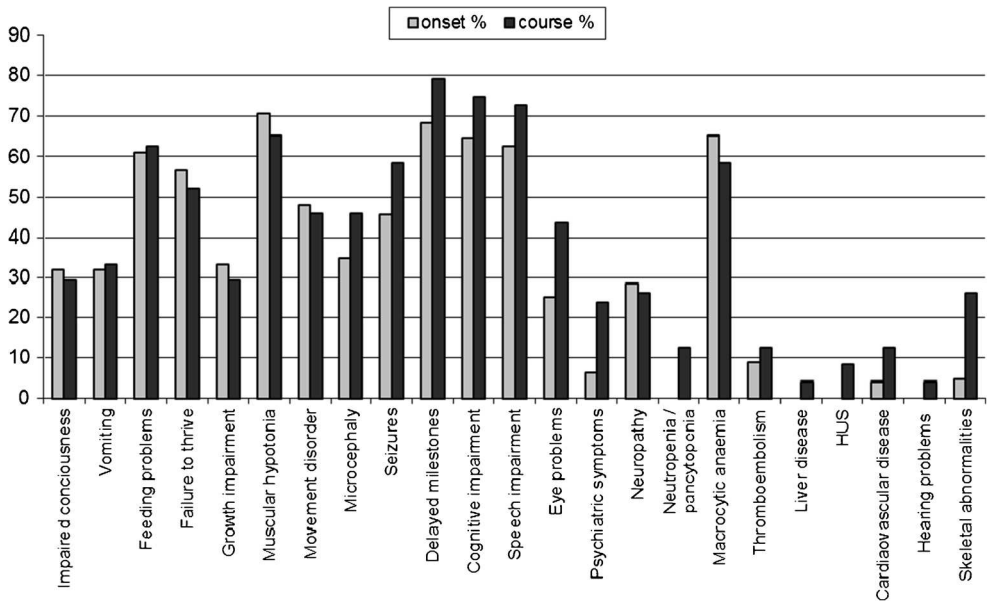

Fig. 2 Clinical presentation at onset and during the course of the disease (%) in 24 individuals with the cblE and cblG defect

retinopathy, nystagmus and strabismus; lens dislocation was never observed.

CNS involvement was also evident in magnetic resonance imaging studies, which were available for 22 patients. Findings were normal in only four patients. Enlarged ventricles or subarachnoidal spaces were described in 3/22, brain atrophy in 10/22 and white matter changes in 8/22 patients. In one patient, cerebellar atrophy and in another multiple morphologic brain malformations (vermis hypoplasia, thin corpus callosum and hydrocephalus) was present. Hydrocephalus was encountered in four patients: one cblG patient had presented with facial dysmorphic features, ureteral duplication and external hydrocephalus at birth, another with hydrocephalus and cerebral and optic nerve atrophy at age 2 months and in two children internal hydrocephalus was observed at month 3 (cblE) and 7 (cblG), respectively. An overview of the main clinical features observed in each individual patient is depicted in Table 1.

Among the more rarely reported symptoms were sinus vein thrombosis and deep vein thrombosis of the lower leg, occurring each in a single patient. The deceased cblE patient had developed ischemic–haemorrhagic basal ganglia lesions and HUS during the course. A milder episode of HUS was recorded in a cblG patient at the age of two years. Liver disease was reported in a single patient in terms of a non-specific discrete elevation of bilirubin probably not related to the remethylation defect but to a more frequent condition such as Gilbert-Meulengracht syndrome. Skeletal abnormalities (e.g. osteoporosis, scoliosis) were mostly attributable to severe neurological disease.

Outcome and quality of life (QOL)

At the latest time of follow-up, two patients attended Kindergarten; four a regular school, two a special school due to impaired motor functions and 12 individuals required special schooling due to impaired cognitive function. One subject worked in a sheltered employment company. Twenty-one individuals lived with their parents; one adult patient required sheltered housing. Seventeen individuals were able to walk without assistance and eating without assistance was possible in 15 cases. In contrast, only seven subjects were able to communicate without impairment, learning disabilities were noticed in 15, behavioural problems in 11 and social problems in nine patients. For assessment of QOL in patients and caregivers, burden of treatment and impact of treatment on biochemical and clinical parameters, as well as to obtain an overall impression of the severity of handicap at the latest follow-up, physicians were asked to give their overall estimations on a 5-point Likert scale ranging from "strongly agree" to "strongly disagree" (Supplementary Fig. 3). Fifty-seven percent of physicians agreed or strongly agreed to the statement "the patient is severely handicapped"; however, 64 and 74 % stated that their patients showed good biochemical and clinical response to treatment. Patients' and caregivers' quality of life was estimated as "good" in 73 and 57 % of responses respectively. Treatment was considered a "significant burden" for the patient in 29 % of cases.

Additionally, relations between physicians' overall ratings of clinical and biochemical response, patients' and caregivers' QOL, burden of treatment for the patient and outcome and age-related parameters as well as signs and symptoms at onset and during the course of the disease were investigated. Regarding outcome, early disease onset was significantly associated with severe handicap, learning disabilities, social problems, a higher frequency of neuro-ophthalmological problems as well as lower proxy-rated patients' and caregivers' QOL. In the long term, impaired ability to communicate was seen more frequently in patients with longer delay to diagnosis ($p<0.05$; Spearman rho; Supplementary Table 1).

Treatment

Biochemical response to treatment was evident in all patients. Mean tHcy at diagnosis was 140 μmol/L (range: 53–279, median 125.8 μmol/L) compared to 71 μmol/L (range 9–141, median 77 μmol/L) under treatment. Met was low at diagnosis (mean 10.9 μmol/L; range: 0–28, median 9 μmol/L) and increased under treatment (mean 32.9 μmol/L; range: 14–85, median 27.5 μmol/L). Treatment was variable especially concerning type of cobalamin preparations and the doses of medications (Table 2).

Biochemical and molecular genetic characterization and clinical parameters

Results of biochemical and molecular genetic characterization are summarized in Supplementary Table 4. Allele frequencies are summarized in Supplementary Table 5.

Enzymatic studies performed for diagnostic purposes showed deficient methionine formation and cobalamin coenzyme synthesis consistent with the underlying defect in all patients. Responsiveness of methionine synthesis to supplementation of medium with OH-Cbl was observed in all cell lines with a tendency towards higher levels in supplemented medium for cblG compared with cblE lines. When comparing individual clinical courses and outcome data with the results of enzymatic studies in cultivated fibroblasts, no general/obvious correlation between both data sets could be delineated. Likewise, no relation between genotype and clinical phenotype was observed. Molecular genetic investigation revealed a majority of private mutations without predominance for any

Table 1 Clinical features in 11 patients with the cblE defect and 13 patients with the cblG defect ([z]d=days; m=months; y=years)

ID	Age at onset[z]	Age at last assessment[z]	Time from first symptoms to treatment[z]	Main symptoms at onset								Course of disease/ status at last assessment
				Feeding problems	Seizures	Muscular hypotonia	Dev. delay	Impaired consciousness	Macrocytic anaemia	Other symptoms	Reported brain MRI findings	
cblE												
16[*]	43 d	56d	1 d	x						HUS	Ischaemic-haemorrhagic basal ganglia lesions; white matter decrease in the grooves	Deteriorating / deceased
24[$]	3 m	22 m	days	x	x	X	x	x		Neutropenia	Delayed myelination, diffuse white matter loss at age 8 m	Stable / dev. delay
20	3 m	17y	> 7 y		x		x		x		Hydrocephalus vermis hypoplasia; thinned corpus callosum; white matter volume loss	Stable / seizures, dev. delay
18	14 d	10y	days	x		x	x		x		Normal	Stable / severely handicapped
10[#]	1 m	20y	4 m	x					x	Nystagmus, muscular hypertonia	White matter changes at age 5y	Improved / severe visual impairment
4	2.5 y	16y	>10 y			x	x		x		Normal	Stable / learning and behavioural problems
3[+]	1st day	29 m	2 m	x	x	x		x		Metabolic crisis, arterial hypertension	Brain atrophy	Stable / severely handicapped
17[&]	3 y	22y	days						x		T2 flair hyperintensities in globus pallidus and left thalamus	Stable / normal
8[#]	1 m	32y	8 y	x	x	x	x		x	Movement disorder	Normal at age 31 y	Stable / severely handicapped
11	3 y	11y	days						x		n.d.	Stable / normal
22	Unknown	22y	years		x	x				Neuropathy, movement disorder, venous thrombosis	Brain atrophy; periventricular hyperintensities	Improved / severely handicapped
cblG												
23	3 m	11 y	6 m	x		x	x	x	x		Brain atrophy	Stable / severely handicapped
5	2 m	6 y	1 m		x	x	x	x		Strabismus, nystagmus	Brain atrophy, demyelinated areas; white matter edema Hydrocephalus (7 m)	Improved / severely handicapped
15	1st d	10 y	8 m	x		x		x	x		Brain atrophy	Improved / severely handicapped
7	2 m	12 m	2 m	x			x		x	Neuropathy, movement disorder	Mild enlargement of subarachnoidal space	Improved / severely handicapped
6	3 m	16 m	days	x	x		x		x		Normal at age 4 m	Stable / severely handicapped
12	17 y	20 y	days	x					x		n.d.	Stable / normal
19	2 m	4 ½ y	1 m	x		x				Dysmorphic features	Enlarged subarachnoidal spaces; brain atrophy	Improved / developmental retardation
1	10 d	15 y	3 m	x		x				optic nerve atrophy	Hydrocephalus; brain atrophy	Stable / severely handicapped,

Table 1 (continued)

ID	Age at onset[r]	Age at last assessment[r]	Time from first symptoms to treatment[r]	Main symptoms at onset					Other symptoms	Reported brain MRI findings	Course of disease/ status at last assessment
				Feeding problems	Seizures	Muscular hypotonia	Dev. delay	Impaired consciousness	Macrocytic anaemia		
14	21 d	12 y	4 m	x	x	x	x	x	x	Ataxia	Brain atrophy; white matter changes	Stable / learning & behaviour problems
2	5 m	2 ½ y	3 m	x	x	x					Discrete enlargement of ventricles and subarachnoidal space	Normal
9	3 m	7 y	4 y	x		x	x		x		Mild diffuse white matter changes at age 4.5y	Stable / severely handicapped
13	8 m	13	>10 y		x		x		x	Neuropathy	Brain atrophy; periventricular white matter changes	Stable / severely handicapped
21	15 m	6 ¾ y	18 m			x	x		x	Sinus venosus thrombosis Ataxia, mild HUS	Mild brain atrophy; hydrocephalus after sinus vein thrombosis	Stable / moderately handicapped

Patients published before in *(Palanca et al 2013); [$] (Kandula et al 2014); [#] (Zavadakova et al 2002), (Zavadakova et al 2005); [&] (Vilaseca et al 2003), (Zavadakova et al 2005); [+] (Zavadakova et al 2005), (Müller et al 2007)

single mutation or genotype. Eight novel mutations in *MTRR* and 15 novel mutations in *MTR* were identified.

Discussion

In this retrospective survey on 24 individuals with the cblE or cblG defect, signs and symptoms at onset and during the course were recorded. No difference concerning the frequency of clinical symptoms at any time was noted between cblE and cblG patients except for muscular hypotonia during the course, which is more frequently reported in patients with the cblG defect. Due to this almost complete clinical overlap, both disorders have been evaluated together.

Clinical features in the cohort are generally similar to those published. The new information provided by the study is generated by the investigation of symptoms both at onset and during the course, MRI findings, outcome and everyday abilities as well as QOL combined with enzymatic studies and molecular genetic characterization of the patients.

Nevertheless, we are aware of the limitations of this retrospective survey study, which is with respect to the clinical information which is based on proxy reports by physicians and not on self-reported patient and caregiver's observations.

Prenatal and birth-related problems are uncommon in the cblE and cblG defects. Disease onset occurs in 92 % of patients before the age of 3 years with a peak in the first year of life where already 75 % of patients are symptomatic. The characteristic clinical pattern encompasses feeding difficulties and failure to thrive, macrocytic anaemia, neurocognitive impairment and eye disease (nystagmus, abnormal eye movements, impaired visual acuity, and strabismus). White matter disease and brain atrophy are common features in the reported cohort, thus supporting the hypothesis that reduced availability of Met results in decreased S-adenosylmethionine availability and consecutively in an impairment of methylation capacity, which has been related to hypomyelination in the central nervous system (Surtees 1998).

Relatively rare manifestations in this cohort include hydrocephalus and HUS with four and three affected patients, respectively. Renal failure following HUS occurred in the deceased patient and in a milder form in another patient. HUS associated with a fatal course of the disease has been reported in another young child (Palanca et al 2013). Metabolic crises are not a predominant feature of the disease either at presentation or during the course and unscheduled admissions became only necessary in less than one third of the patients.

Two patients presented with the mild cblE phenotype with only macrocytic anaemia in the absence of neurological symptoms. One was homozygous for the c.1361C>T mutation in the MTRR gene as described before (Vilaseca et al 2003) but another patient was compound heterozygous for

Table 2 Treatment in the cohort of 24 cblE and cblG patients

	N	Range mg/week	Mean mg/week	Median mg/week
OH-Cbl IM	10	0.25–20	7.9	6
OH-Cbl IV	2	2–7	4.5	–
OH-Cbl PO	3	1–7	5	7
OH-Cbl combined IM/PO	1	24.5	–	–
Cyano-Cbl PO	5	0.25–3.5	1.1	0.5
Methyl-Cbl PO*	1	14	–	–
5′Deoxyadenosyl-Cbl PO	1	14	–	–
	N	Range mg/kg/d	Mean mg/kg/d	Median mg/kg/d
Betaine	22	51–282	161	185
	N	Range mg/d	Mean mg/d	Median mg/d
Folate	14	1.25–40	7.6	5
Folinate	9	1–45	13.5	7.5
Methionine	4	80–500	256	200

*In a non-responsive patient 1 mg OH-Cbl/week i.m. was changed to oral methyl-Cbl treatment

c.1678_1681del and c.1740C>G, thus indicating more than one genotype being associated with this milder course of the disease. Due to the large number of private mutations, no clear genotype-phenotype correlation could be identified. Although not a main aim of this study there appears to be no obvious connection of the functional enzyme parameters we measured in cultivated fibroblasts with clinical presentation, course or outcome.

Like in other inborn errors of cobalamin metabolism, delay in diagnosis and consequently time to initiation of adequate treatment is significant. We investigated which parameters correlate with a shorter interval between onset of symptoms and diagnosis. A "typical metabolic" presentation with feeding problems and altered consciousness in a very young child prompts metabolic workup and leads to the correct diagnosis more rapidly than the less uniform presentation in patients with later onset disease. In the present cohort, delayed diagnosis correlates with the outcome parameter "reduced ability to communicate" but not with other outcome parameters or proxy-reported QOL.

Good response to early treatment in remethylation disorders has been reported before (Müller et al 2007; Schiff et al 2011). However, in this study statistical analyses revealed no difference between the observed frequency of symptoms at onset and during the course of the disease. Beyond this, analysis of the neuro-ophthalmological and psychiatric symptoms as combined in a single factor representing overall CNS involvement revealed deterioration over time despite treatment. These findings underline the severity of the diseases which is also supported by the finding of a very high incidence (18/22 patients) of pathological brain MRI findings as well as cognitive, behavioural and social problems. One patient was deceased and 57 % of the patients were considered "severely handicapped". However, physicians gave the overall judgement that their patients mostly benefit from treatment and considered the course of the disease to be improving or stable in all survivors. We speculate that physicians might have focused predominantly on the improving biochemical parameters (tHcy, Met) and the non-CNS parameters such as haematological problems and failure to thrive/feeding problems which remain stable or improve slightly. Additionally, the progression of CNS abnormalities may in part simply reflect the fact that some symptoms can only be attributed to and assessed in older children (e.g. psychiatric and behavioural issues).

Treatment strategies in cblE and cblG patients are very variable in terms of different Cbl preparations and applications as well as the large variation of doses for all medications. Methionine was supplemented only in four patients; the majority of patients were treated with Cbl, folate or folinate and betaine. THcy was lowered and Met increased in all treated patients but the range of tHcy concentrations (maximum 141 μmol/L) suggests that optimal doses and forms of application might not have been achieved in all cases. How to determine the optimal dosage in the intracellular cobalamin disorders is an unsolved issue at present. In patients with the cblC defect, some authors argue in favour of general high-dose OH-Cbl treatment (Carrillo-Carrasco et al 2009) while others advise individual titration of the dose targeting optimal biochemical response (Dionisi-Vici et al 2013). Since parenteral Cbl application is necessary in most cases, the number of injections per week is an important issue for patients' and caregiver's QOL (Dionisi-Vici et al 2013). It has not been investigated systematically whether higher doses in general are advantageous and if so, whether single high compared to repeated lower doses changes clinical outcome in any of the cobalamin defects effectively. Future studies addressing this question in a standardized manner are needed. In general, it is

difficult to evaluate the efficacy of treatment strategies in the context of a disease highly variable in its clinical manifestations and genetic backgrounds. In addition, no standardized follow-up strategies have been agreed on so far.

Factors determining proxy-rated QOL of life must be interpreted cautiously. Due to the method used, our data might predominantly depict physicians' attitudes towards QOL. Therefore, we limit the interpretation of the data to the observations that in general, physicians assigned good overall QOL to patients and caregivers even though they considered their patients significantly handicapped. Besides failure to thrive, feeding problems and vomiting, predominantly neurocognitive factors such as delayed achievement of milestones, impaired speech and communication skills, seizures, psychiatric problems and severe handicap correlate most significantly with the perception of impaired QOL in patients and caregivers.

In conclusion, this systematic study of the largest series of patients so far, confirms that the cblE and cblG defects are severe diseases with onset in the first years of life, poor neurocognitive outcome and significant brain pathology. There is wide overlap with the cblC defect, especially in patients presenting with HUS, hydrocephalus or retinopathy. In the cblC defect it has been hypothesized that the microangiopathy which underlies both HUS and pulmonary arterial hypertension may be related to mutations at a specific site, namely the c.276 nucleotide (Kömhoff 2013). In addition, chronic communicating hydrocephalus may also be related to vasculopathy in terms of reduced intracranial vessel compliance that results in enhanced intracerebral pulse pressure thus causing ventricle enlargement (Greitz 2007). However, in the cblE and cblG defects no specific mutations/mutation sites causing microangiopathy have yet been identified. Generally, no genotype-phenotype or in vitro enzyme activity-phenotype correlations can be derived in the cblE and cblG defects. The exception seems to be homozygosity for the c.1361C>T mutation in the *MTRR* gene, which may be more clearly associated with an isolated macrocytic anaemia phenotype than other genotypes (Vilaseca et al 2003). The clinical course may be stabilized or slightly improved but not reversed by treatment. Treatment strategies are variable and this study points to the need to devise a consensus on preparations and applications of cobalamin, drug dosages or standardized follow-up strategies. Delay in diagnosis is significant, depends on the age and clinical pattern at disease onset and correlates with communicative abilities of the patient in the long-term.

Acknowledgments We thank all patients and their families who gave informed consent to include their data. This work was supported by the Swiss National Science Foundation [grant number 31003A_138521 to M.R.B. and B.F.] and the Rare Disease Initiative Zurich (radiz), a clinical research priority program for rare diseases of the University of Zurich, Switzerland. Institutional support to P.J and V.K. was provided by projects RVO VFN64165 and PRVOUK-P24/LF1/3.

Compliance with ethics guidelines

Conflict of interest None.

Human and animal rights and informed consent All procedures followed were in accordance with the ethical standards of theresponsible committee on human experimentation (institutional and national) and with the HelsinkiDeclaration of 1975, as revised in 2005. Informed consent was obtained from all patients for beingincluded in the study.

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